Light and drug dosimetry considerations in porphyrin precursor–based photodynamic therapy

by

Even Angell-Petersen

Department for Surgery, Section for Surgical Oncology
The Norwegian Radium Hospital
University of Oslo

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo.
No. 633

ISSN 1501-7710

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2007.

Produced in co-operation with Unipub AS.
The thesis is produced by Unipub AS merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

Unipub AS is owned by
The University Foundation for Student Life (SiO)

Acknowledgements

This work was performed at Department of Surgical Oncology and Department of Radiation Biology at the Norwegian Radium Hospital. I am grateful to my supervisor Harald B. Steen for introducing me to biomedical optics, for his advice in scientific writing, and for sharing his instrumentation skills. Furthermore, my work would not have been possible without the unfaltering support from my co-supervisor and department head Karl-Erik Giercksky. I would also like to thank my co-supervisor Johan Moan, the founder of basic PDT research at our institution. As a physicist dealing with medical science I am particularly grateful to both Trond Warloe—my good friend and closest collaborator at the clinic—and Ana Soler, who have shown me how PDT is used in practice to treat patients. Such insight is mandatory for clinical studies and encouraging for experimental studies. Special thanks also goes to Henry Hirschberg for recruiting me to the experimental studies on brain tumors and for enriching my work with his progressive supervision.

I have much enjoyed cooperating with the coauthors Steen Madsen, Qian Peng, Signe Spetalen, Roar Sørensen, Christoph Müller and Cathrine Christensen. Research nurse Anneli B. Madsen has been crucial for the clinical studies, as have the involved employees at PhotoCure ASA. I owe a lot to Dag R. Sørensen for providing the BDIX/BT_4C rat model, giving me training in animal work, and placing his facilities at Center for Comparative Medicine at Rikshospitalet at disposal. Collecting the results in this thesis has also depended on excellent technical assistance from Marie-Therese Strand, Mai Nguyen, Vladimir Iani, and Cecilie Hansen, as well as Jørn Iversen and his staff at the instrument workshop.

Working at Radiumhospitalet has been made pleasant by colleagues such as Jostein Dahle, Pål Kristian Selbo, Sigrid Kvaal, Kristian Berg, and all the other nice people at Department for Radiation Biology and at the outpatient clinic. Moreover, coming home from work is a joy thanks to my dearest friend and wife Kari, who I also thank for proofreading this thesis (including the acknowledgements…). Finally, I send a greeting to Otto, who by being born offered a most welcome two-week break from the tiresome work of finalizing this thesis.

Oslo, June 2007

Even Angell-Petersen

Contents

Abbreviations and symbols:

AK	Actinic keratosis
ALA	Aminolevulinic acid
BAT	Brain adjacent to tumor
BCC	Basal cell carcinoma
CCD	Charge coupled device
H&E	Hematoxylin and eosin
i.p.	intraperitoneal
LED	Light emitting diode
MAL	Methyl aminolevulinate
PDT	Photodynamic therapy
PpIX	Protoporphyrin IX
SD	Standard deviation
SEM	Standard error of the mean

$^1O_2$	Singlet oxygen
ϕ	Light fluence rate (mW cm^{-2})
E	Irradiance (mW cm^{-2})
P	Radiant power (mW)
μ_a	Absorption coefficient (cm^{-1})
μ_s'	Reduced scattering coefficient (cm^{-1})
δ	Light penetration depth (mm)

Glossary:

Action spectrum Spectrum describing for which wavelengths a certain process will take place.
Apoptosis Cell death controlled actively by a "suicide" program.
Carcinoma Malignant tumor originating from epithelial tissues
Cytoreduction Decrease in the number of (tumor) cells.
Dysplasia A pre-cancerous change.
Endogenous Originating within the organism or cell.
Endothelium The epithelium lining the interior surface of blood vessels.
Exogenous Originating outside the organism.
Glioma Tumor originating from glial cells, the major non-neuronal cell type in the central nervous system.
Gray matter Regions of brain and spinal chord consisting of mainly neurons, cell bodies, and capillaries.
Indwelling Placed or implanted within the body, as a catheter or electrode.
Inoculation Introduction (of for instance tumor cells) into a host.
Necrosis Death of cells or tissues as a result of external damage.
Neutrophils Subtype of white blood cells that is much involved in the acute phase of inflammation.
Occlusive dressing An air- and watertight dressing.
Photobleaching Degradation of a dye as a result of light exposure.
Pro-drug An inactive substance that is converted to a drug within the body.
Quantum yield The probability that an excitation will lead to a certain process.
Squamous cell carcinoma *in situ* (or Bowen's disease) An early noninvasive stage of cancer originating from the squamous cells in skin or mucosa.
Systemic application Distribution of a substance throughout the whole body
Thermal penetration depth Parameter related to the range of a local temperature aberration in a tissue. (Definition in publication IV.)
Turbid Cloudy or hazy.
White matter Regions of brain and spinal chord consisting mainly of neural fibers.

List of publications

I. Soler A.M., Angell-Petersen E., Warloe T., Tausjø J., Steen H.B., Moan J. and Giercksky K.E. (2000): Photodynamic therapy of superficial basal cell carcinoma with 5-aminolevulinic acid with dimethylsulfoxide and ethylendiaminetetraacetic acid: a comparison of two light sources. *Photochemistry and Photobiology* **71**: 724–729

II. Angell-Petersen E., Sørensen R., Warloe T., Soler A.M., Moan J., Peng Q. and Giercksky K.E. (2006): Porphyrin formation in actinic keratosis and basal cell carcinoma after topical application of methyl 5-aminolevulinate. *Journal of Investigative Dermatology* **126**: 265–271

III. Angell-Petersen E., Christensen C., Müller C.R. and Warloe T. (2007): Phototoxic reaction and porphyrin fluorescence in skin after topical application of methyl aminolevulinate. *British Journal of Dermatology* **156**: 301–307

IV. Angell-Petersen E., Hirschberg H. and Madsen S.J. (2007): Determination of fluence rate and temperature distributions in the rat brain; implications for photodynamic therapy. *Journal of Biomedical Optics* **12**: 014003-1–014003-9

V. Angell-Petersen E., Spetalen S., Madsen S.J., Sun C.H., Peng Q., Carper S.W., Sioud M. and Hirschberg H. (2006): Influence of light fluence rate on the effects of photodynamic therapy in an orthotopic rat glioma model. *Journal of Neurosurgery* **104**: 109–117

VI. Madsen S.J., Angell-Petersen E., Spetalen S., Carper S.W., Ziegler S.A. and Hirschberg H. (2006): Photodynamic therapy of newly implanted glioma cells in the rat brain. *Lasers in Surgery and Medicine* **38**: 540–548.

1 Introduction

Photodynamic therapy (PDT) is mediated by a photosensitizer—a substance able to absorb energy from light and subsequently transfer it to oxygen, which in its turn deposits the energy as damage to biological matter. Consequently, the treatment involves both distribution of a drug (the photosensitizer) and distribution of light using an adequate light source. The knowledge of photosensitizing drugs is ancient, but it is mainly throughout the last 30 years that PDT has been developed as a treatment modality for cancer (Ackroyd *et al.* 2001). A range of photosensitizers are currently approved for various indications (Figure 1).

1.1 The fundamentals of PDT

A good photosensitizer must absorb light efficiently. A molecule absorbs light by being excited to a different state, gaining the energy of one photon (Einstein 1912). Molecules with chains of conjugated bonds can have electron states matching the photon energy of visible or near infrared light. The skeleton of most sensitizers used

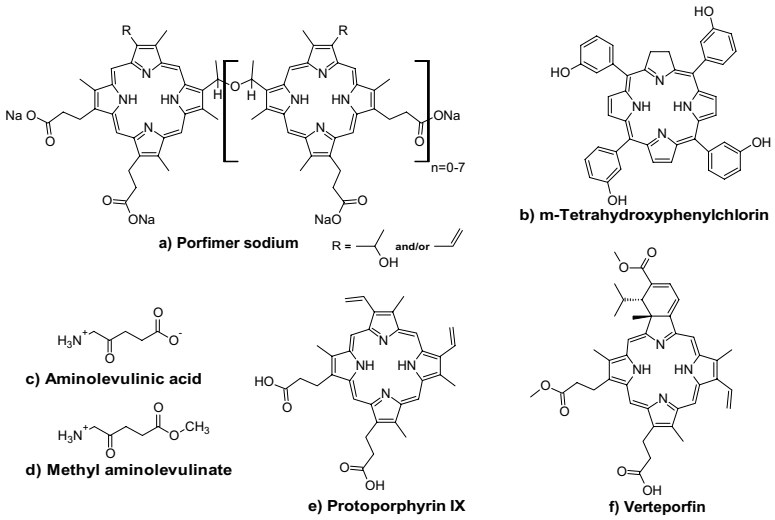

Figure 1: Chemical structure of photosensitizers approved by medical authorities for treatment of (a) esophageal dysplasia, (b) head and neck cancer, (c and d) actinic keratosis, (d) non melanotic skin cancer, and (f) wet macular degeneration. The prodrugs (c) and (d) are synthesized into (e) intracellularly.

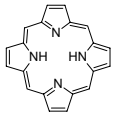

Figure 2: Porphin

in PDT is the porphin (Figure 2), which consists of four pyrrole rings joined to a ring structure with a cyclic, conjugated system of 18 π-electrons. This system has electron states matching visible light. Molecules in excited electron states may participate in chemical reactions damaging biomolecules that are vital for the function of a cell. Generally speaking, such reactions do not depend on the presence of oxygen, but photodynamic effect is defined as oxygen-dependent reactions exclusively (Blum 1941).

Most states excited by photoexcitation are short-lived (a few nanoseconds), and will return to the ground state by fluorescence or heat-transfer before interaction with other molecules can occur. For most molecules, both the ground state and the first excited state are so-called singlet states, with all electrons paired so that the only option for the molecule's net spin is zero. In general, but almost exclusively in excited states, molecules can also be in triplet states. Two electrons are then unpaired, leaving three options for the net spin component: +1, zero, or –1 (as each electron has spin +½ or –½). Processes not conserving the net spin, such as transfer from an excited triplet state to a singlet ground state, are relatively unlikely. This means that excited triplet states can be sufficiently stable to allow interaction with other molecules. Photodynamic effect depends on a high quantum yield for formation of such metastable excited triplet states after photoexcitation (Figure 3 a and b). For good photosensitizers this quantum yield is typically 0.2–0.9 (Redmond & Gamlin 1999).

The reactions dominating for most sensitizers used in PDT are by the so-called *type II* pathway, where the excited triplet state ($^3PS^*$) interacts with molecular oxygen by energy transfer. The sensitizer returns to the singlet ground state (^1PS), and oxygen is excited to the reactive species *singlet oxygen* ($^1O_2$) (Figure 3c) (Kawaoka *et al.* 1967). As illustrated in Figure 3c, oxygen's unique feature as a molecule with a triplet ground state ($^3O_2$) makes this a spin-conserving process, which is more efficient than non spin-conserving reactions with other molecules. Good photosensitizers typically have quantum yields for $^1O_2$ formation in the range 0.1–0.8 (Redmond & Gamlin 1999). Singlet oxygen can return to the ground state by phosphorescence. This is a very slow process, so in non-gaseous environments

$$^1PS(\uparrow\downarrow) + h\nu \rightarrow {}^1PS^*(\uparrow\downarrow) \quad \text{(a)}$$

$$^1PS^*(\uparrow\downarrow) \rightarrow {}^3PS^*(\downarrow\downarrow) \quad \text{(b)}$$

$$^3PS^*(\downarrow\downarrow) + {}^3O_2(\uparrow\uparrow) \rightarrow {}^1PS(\uparrow\downarrow) + {}^1O_2{}^*(\uparrow\downarrow) \quad \text{(c)}$$

Figure 3: (a) Photoexcitation of photosensitizer (*PS*) to excited singlet state. As shown by the arrows both states have paired electrons. (b) Intersystem crossing to a metastable excited triple state that has unpaired electrons with parallel spins. (c) The *type II* reaction, were the metastable triple state excites ground state triplet oxygen into singlet oxygen by energy transfer.

participation in chemical reactions is much more likely. $^1O_2$ reacts with a variety of biomolecules, and these reactions are so efficient that the intracellular lifetime of $^1O_2$ is in the nanosecond range, corresponding to a radius of action around 0.01 microns (Moan & Berg 1991, Niedre *et al.* 2002). This means that most of the initially induced damage is restricted to the subcellular compartment containing the photosensitizer. Consequently, when distributing the photosensitizer, intracellular localization may be as important as concentration. Reported estimates of the number of $^1O_2$ molecules necessary to kill a cell has varied with three orders of magnitude from 10^8 to 10^{11} in different experimental setups (Niedre *et al.* 2005). There is some uncertainty in these estimates, but the vast range reflects the fact that PDT inactivation is not necessarily a simple matter of acute lethal $^1O_2$ damage. A thorough understanding of the biology is necessary to describe the PDT-induced damage, which may be affected significantly by factors such as cell signaling pathways, programmed cell death, indirect vascular damage, and effects of the host's immune system (Dougherty *et al.* 1998).

Photochemical reactions can also be mediated by the *type I* pathway, where (unlike Figure 3c) the excited triplet state interacts with a substrate by electron or proton transfer. This leads to generation of cytotoxic agents, including reactive oxygen species other than $^1O_2$. The prevailing hypothesis is that $^1O_2$ is the important cytotoxin in PDT (Weishaupt *et al.* 1976, Fuchs & Thiele 1998), although additional effects of *type I* reactions can not be discounted. It has been reported that reactive oxygen species other than $^1O_2$ can dominate for certain sensitizers and environments (Vakrat-Haglili *et al.* 2005). However, for protoporphyrin IX mediated PDT – the topic of this thesis – it has recently been shown by direct *in vivo* measurements of $^1O_2$ phosphorescence that the PDT-induced damage is directly related to the amount of $^1O_2$ produced (Niedre *et al.* 2005).

1.2 Drug distribution

Most photosensitizers used in PDT are applied systemically by intravenous injection. Different photosensitizers, formulations and application schemes can target different tissues. Typically, short drug–light intervals (down to minutes) lead to vascular damage, while direct damage to tumor cells can be achieved using longer drug–light intervals (up to several days). The localization is also strongly dependant on the physiochemical properties of the drug, and in some cases of its ability to bind to specific targets in endothelial or tumor cells (Chen *et al.* 2006). The photosensitizer, the drug administration, and the drug–light interval should ideally be optimized to achieve sufficiently high accumulation of photosensitizer in the target tissue and acceptably low accumulation in the surrounding normal tissues. Properties of tumors that make selective photosensitizer uptake possible include: a leaky vascular system,

poor lymph drainage, large extracellular volume, high amounts of collagen, decreased pH, and deviating cellular receptors and uptake mechanisms (Dougherty *et al.* 1998). Still, the selectivity achieved with systemic photosensitizers is often not better than around a factor of two (Moore *et al.* 1997). A major exception is brain tumors, where at least one order of magnitude of selectivity can be achieved for photosensitizers that to a low degree penetrate the blood–brain barrier (Chopp *et al.* 1996, Obwegeser *et al.* 1998, Lobel *et al.* 2001). Maximum selectivity is always desirable, but the degree of damage that can be tolerated in the surrounding normal tissues varies substantially. In some cases—like for m-tetrahydroxyphenylchlorin mediated PDT for head and neck cancer—severe necrosis following PDT can heal up well without unacceptable scarring (Hopper *et al.* 2004). In other areas—like the esophagus, brain, or retina—damage to normal tissue can be dangerous.

1.2.1 Porphyrin precursor mediated PDT

As an alternative to direct administration of a photosensitizer, it is possible to make an organism photosensitive by administering drugs that are not photosensitizers themselves. The naturally occurring compound aminolevulinic acid (ALA) can be used as a pro-drug to induce accumulation of endogenous porphyrins that act as photosensitizers (Peng *et al.* 1987, Malik & Lugaci 1987). The porphyrins are synthesized intracellularly by the heme synthesis pathway as shown in Figure 4. The rate

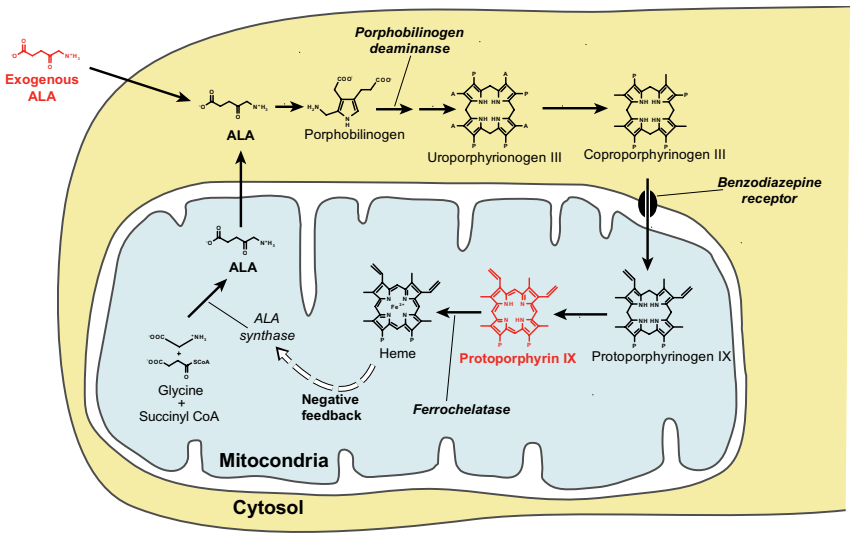

Figure 4: The heme biosynthesis that takes place in eukaryotic cells. Excess of ALA will lead to accumulation of PpIX, the immediate precursor to heme. For several neoplastic cell types this accumulation is enhanced due to increased activity of porphobilinogen deaminase, decreased activity of ferrochelatase, or increased transport past the mitochondrial membrane due to abundant expression of the peripheral benzodiazepine receptor (Collaud *et al.* 2004).

limiting step of this pathway is the synthesis of ALA. If an excess of ALA is supplied exogenously, the insertion of iron into the porphyrin ring to form heme becomes the rate limiting step. This leads to accumulation of the photosensitizer protoporphyrin IX (PpIX).

Systemic application of ALA is possible and is used for experimental therapy of various indications including esophageal dysplasia (Pech *et al.* 2005), but local drug application with topical formulations is most common. Local drug application is a great advantage as it eliminates the obvious problems associated with general photosensitization of the whole patient. Topical ALA PDT was introduced by Kennedy *et al.* in 1990. Since then, a variety of skin disorders have been treated using ALA or other porphyrin precursors. The transport of ALA through tissues and into cells has been reported to be suboptimal (Peng *et al.* 1995, Martin *et al.* 1995, Wolf & Kerl 1995). ALA esters, which are more lipophilic porphyrin precursors, were introduced to overcome this limitation and to improve the bioavailability (Peng *et al.* 1996, Kloek & Beijersbergen 1996). Differences between ALA and its esters have been shown for a variety of key issues, including uptake and porphyrin production in cells (Gaullier *et al.* 1997, Rud *et al.* 2000, Gederaas *et al.* 2001, Perotti *et al.* 2004), transport through tissues (de Rosa & Bentley 2000, Bigelow *et al.* 2001, van den Akker *et al.* 2003), ability to induce porphyrin production in tissues (Fritsch *et al.* 1998, Lange *et al.* 1999, Perotti *et al.* 2002), effects of drug formulations (Casas *et al.* 2001, Lopez *et al.* 2004), systemic uptake (Moan *et al.* 2001), and pain felt during PDT (Wiegell *et al.* 2003, Kasche *et al.* 2006).

A vide variety of tissues are photosensitized by porphyrin precursors, but certain tissue types have a low ability to produce endogenous porphyrins. These include cases where damage would be critical such as connective, muscle, and brain tissues (Fukuda *et al.* 1992). This represents a useful selectivity enhancement, but it can also limit the efficacy of the treatment for certain tumors that has a low ability to produce endogenous porphyrins (Morgan *et al.* 2004). Successful indications for por-

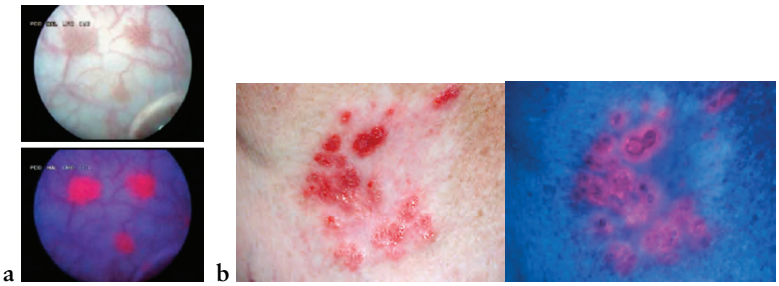

Figure 5: Porphyrin fluorescence from cancer in (a) bladder mucosa (Berg *et al.* 2005) and (b) skin after local application of hexaminolevulinate and methyl aminolevulinate, respectively.

phyrin precursor mediated PDT mostly include premalignant lesions or low-grade non-invasive tumor types. Porphyrin precursors can also be of diagnostic value as a contrast agent, utilizing PpIX's characteristic fluorescence (Figure 5). This is most widely used in urology, where hexaminolevulinate (the hexyl ester of ALA) is approved for fluorescence-guided detection of bladder cancer (Jocham *et al.* 2005). For malignant brain tumors the usefulness of ALA-based, fluorescence-guided resection has recently been demonstrated in an extensive *phase III* clinical trial (Stummer *et al.* 2006).

1.3 Light distribution

The targeted application and limited penetration of light makes PDT a local treatment modality. Transport of light through tissue is limited by absorption and scattering. These processes result in an approximately exponential decrease in the fluence rate (i.e., the light intensity inside the tissue) as the light penetrates tissue. For an infinite planar geometry, the *light penetration depth* (δ) is the distance at which the fluence rate has been reduced by a factor e (≈ 0.37) (Star 1997). As shown in Figure 6a, the light penetration depth is strongly dependent on wavelength. Most porphin based photosensitizers have their absorption maximum in the blue region around 400 nm, but the hemoglobin found in most tissues limits the penetration depth of blue light to about a tenth of a millimeter. The photosensitizers most commonly used in PDT (Figure 1, page 1) absorb also in the red region, where the penetration depth is 2–3 mm for most tissues (Cheong 1995). The best light penetration is found in the near infrared region from about 750 to 850 nm where penetration depths can reach 5–6 mm. The sensitizers motexafin lutetium (732 nm) and Pd-bacteriopheophorbide (TOOKAD®) (762 nm) absorb in this region and are used for experimental PDT of prostate cancer (Zhu *et al.* 2005, Weersink *et al.* 2005).

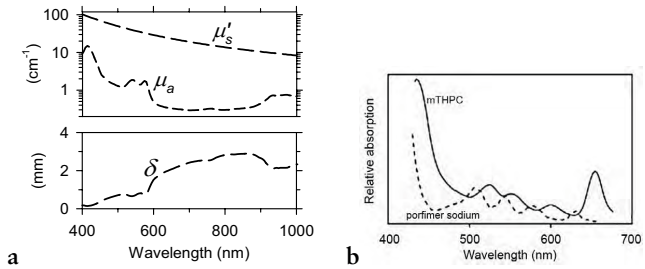

Figure 6: a) Absorption coefficients (μ_a) and reduced scattering coefficients (μ_s') typically found in tissue, based on spectra published by Jacques *et al.* (1998). The resulting light penetration depth (δ) is calculated as $1/\sqrt{3\mu_a(\mu_a + \mu_s')}$. b) Absorption spectra of the photosensitizers porfimer sodium and m-tetrahydroxyphenylchlorin (Triesscheijn *et al.* 2006a).

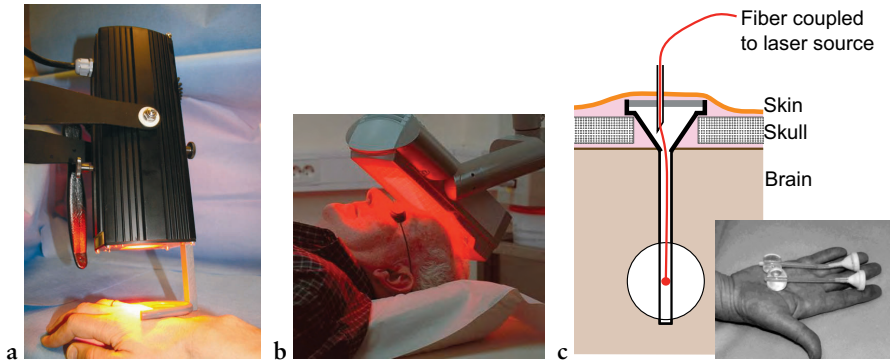

Figure 7: Light sources developed for PDT: (a) a filtered halogen lamp and (b) an LED lamp for light delivery to skin. c) Indwelling balloon applicator for light delivery to the brain (Madsen *et al.* 2001).

1.3.1 Light sources

Vast amounts of light are required during PDT. Tissues are typically exposed to irradiances around 100 mW cm^{-2}, which is comparable to the maximum total solar irradiance in southern Norway (Huld & Suri 2006). The treatment modality has to some extent been limited by the availability of adequate light sources providing sufficient output powers of red light. However, reliable semiconductor-based light sources have become available during the last 5–10 years. Such diode lasers and light emitting diodes (LEDs) are currently the dominating light sources in PDT.

Laser light with a wavelength matching the absorption maximum of the photosensitizer provide the most efficient excitation. When using a laser it is also possible to couple the light into an optical fiber to illuminate sites such as the esophagus, prostate, oral cavity or brain (Figure 7c). In cases where the use of fiber optics is not necessary, sufficient excitation may in many cases be achieved using broadband light sources such as LEDs or filtered incandescent lamps (Figure 7 a and b) (Brancaleon & Moseley 2002). Systems based on arrays of high power LEDs are currently dominating for PDT in dermatology in Europe. Compared to diode lasers, LED systems are cheaper, more compact, and do not represent any risk for retinal injury (ICNIRP 1997).

1.3.2 Light dosimetry

The simplest way to describe the light dosimetry of a PDT session is to specify the amount of light delivered *to* the tissue. When treating a surface, the dose is then specified as the time integral of the irradiance. The irradiance, E, has unit W cm^{-2} and is defined as the radiant power entering a surface, divided by the surface area. When treating with an indwelling applicator, like a fiber tip embedded in the tissue, the dose can be specified as the total radiant energy delivered from the applicator. More sophisticated light dosimetry involves calculating the actual fluence rate distri-

bution inside the tissue. The fluence rate, ϕ, has unit W cm^{-2} and is defined as the radiant power passing through an infinitesimal sphere divided by the sphere's cross-sectional area. The fluence rate distribution for a specific treatment session depends on both the geometry and the optical properties of the tissues. It can be calculated by solving the Boltzmann transport equation, a partial differential equation describing the transport of light in turbid media, or by using Monte Carlo simulation to stochastically imitate the propagation of photons through turbid media (Star 1997). Figure 8 shows examples of calculated fluence rate distributions for two light delivery geometries.

The ultimate dose measure in PDT is the distribution of the $^1O_2$ formation, which depends on the treatment variables light, photosensitizer, and oxygen. However, the distribution of $^1O_2$ is very difficult to calculate since these three factors are far from constant during the light exposure (Wilson *et al.* 1997): The PDT effects alter the blood supply and hence the oxygen supply. The oxygen concentration is also reduced as oxygen is consumed by the production of $^1O_2$. In addition, the photosensitizer concentration is in most cases significantly reduced (photobleached)

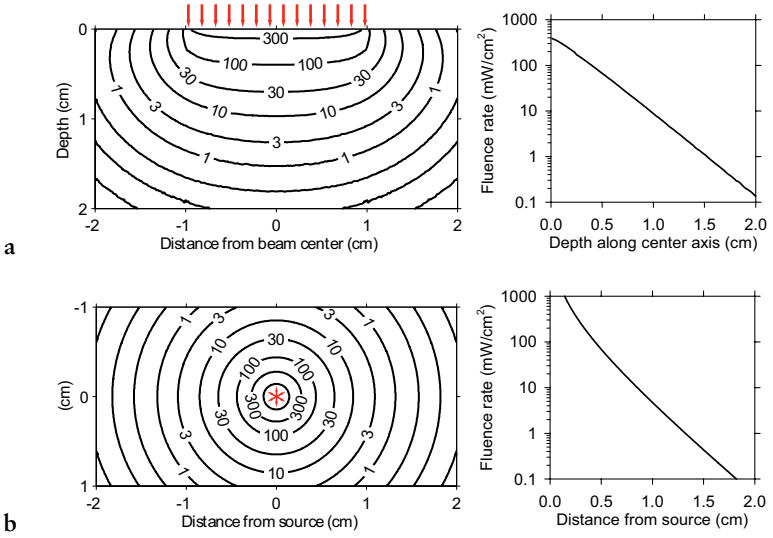

Figure 8: Light distributions calculated for (a) external and (b) interstitial light delivery. Calculations were made for a surface exposed to a 2 cm beam with irradiance 100 mW cm^{-2}, and an infinite medium with an isotropic point source of 100 mW. The fluence rates are visualized as iso–fluence rate contours (unit: mW cm^{-2}) and as a function of depth. For the external delivery the fluence rates were calculated using the Monte Carlo software MCML (Wang *et al.* 1995). For the interstitial delivery the expression $(3P/4\pi)(\mu_s' + \mu_a)e^{-\frac{r}{\delta}}/r$, which is derived using the diffusion approximation of the Boltzmann transport equation, was used (Driver *et al.* 1991). In both cases the optical properties of the medium were set to μ_a = 0.5 cm^{-1}, μ_s' = 10 cm^{-1} and δ = 0.252 cm.

by the presence of light. The change of blood supply, the oxygen consumption, and the photobleaching may depend heavily on the *rate* of the light delivery. To top off the complexity, the optical properties of the tissue depend strongly on blood content, blood oxygenation, and photosensitizer concentration. Due to these difficulties the by far most common dose measure in PDT is a simple specification of the light delivered to the tissue. Calculations of fluence rate distributions are mostly used for general purposes, and are rarely performed for a single patient. Recently it has become possible to assess the $^1O_2$ formation directly by *in vivo* measurement of $^1O_2$ phosphorescence (Niedre *et al.* 2002). Massive technical difficulties must be overcome to introduce such measurements clinically, but they may become a golden standard for PDT dosimetry in the future.

1.4 PDT in dermatology

Topical porphyrin precursor mediated PDT is becoming a widespread treatment modality in dermatology, and the prime indications are currently the non-melanotic skin cancer *basal cell carcinoma* (BCC) and the precancerous skin growth *actinic keratosis* (AK). BCC (Figure 9) is the by far most common malignancy within the Caucasian populations, with incidence rates of 100–700 per 100 000 per year depending on solar exposure (Brooke 2005). However, it metastasizes in less than 0.03% of cases (Lo *et al.* 1991, Wadhera *et al.* 2006) and is rarely dangerous. AK has an even higher incidence, and has the potential to progress to squamous cell carcinoma if untreated (Ortonne 2002). Several efficient treatment options exist for both BCC and AK, but PDT has the advantage of less treatment related morbidity and a better cosmetic result. PDT is particularly advantageous for treatment of large areas of sun-damaged skin with multiple tumors and premalignancies (Braathen *et al.* 2007). Porphyrin precursor mediated PDT is efficient for AK (Szeimies *et al.* 2002, Jeffes 2002) and superficial BCC (Kennedy *et al.* 1990). Good results are also achieved for thicker subtypes of BCC, like nodular BCC, if most of the tumor mass is removed by a debulking procedure prior to the drug application (Soler *et al.* 1999, Soler *et al.* 2001).

Two different drugs are commonly used: ALA and its methyl ester *methyl ami-*

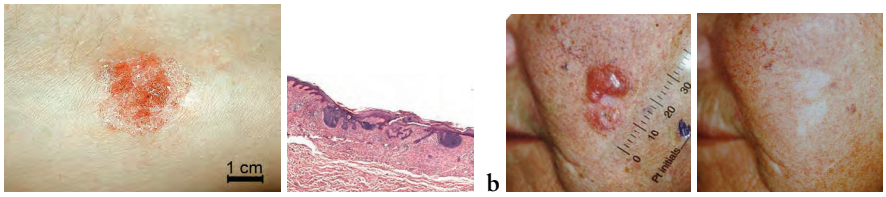

Figure 9: a) Photo and H&E stained histological section of superficial BCC.
b) Nodular BCC before and after MAL PDT.

nolevulinate (MAL). An ALA cream formulation (Levulan Kerastick®, DUSA Pharmaceuticals, USA) is approved in the USA for treatment of AK and is also used cosmetically for skin rejuvenation. Topical ALA PDT has also been shown to be efficient for treatment of superficial BCC and squamous cell carcinoma *in situ* (Braathen *et al.* 2007). An MAL cream formulation (Metvix cream®, PhotoCure ASA, Norway) is approved for treatment of AK (Europe, Australia, USA, Brazil), squamous cell carcinoma *in situ* (Europe), and basal cell carcinoma (Europe, Australia, Brazil). Both MAL and ALA PDT have also recently been proven to have effect on inflammatory acne (Wiegell & Wulf 2006a, 2006b). Comparative clinical studies have shown that MAL leads to more selective porphyrin accumulation (Fritsch *et al.* 1998, Thompson *et al.* 2001) and less pain (Wiegell *et al.* 2003, Kasche *et al.* 2006) than ALA.

Other photosensitizers used successfully to treat non melanotic skin cancer include porfimer sodium (Photofrin®), verteporfin (Visudyne®), and m-tetrahydroxyphenylchlorin (Foscan®) (Lui *et al.* 2004, Oseroff *et al.* 2006, Triesscheijn *et al.* 2006b). These systemically applied drugs can be more powerful than porphyrin precursors, but they are also less selective and cause more side effects and less favorable cosmetic outcomes. Still, they may be beneficial for treatment of large, tick tumors for which porphyrin precursor mediated PDT has a low efficacy.

1.5 PDT of malignant brain tumors

Malignant brain tumors cause 3% of all cancer deaths in Norway (Kreftregisteret 2006). The main treatment modality is surgery, but even if complete resection is achieved patients typically relapse, and cure is rare for most subtypes (CBTRUS 2005). Glioblastoma multiforme (Figure 10), the most common and aggressive subtype, has a median survival of 14 months after a combination of surgery, postoperative radiation therapy, and chemotherapy (Stupp *et al.* 2005). Recurrences often occur from tumor cells invading the normal brain. Although invading tumor cells can be found far from the tumor border, the recurrent tumors are within 2 cm from the resection margin in a majority of the cases (Wallner *et al.* 1989). It is likely that a treatment that eliminates tumor cells embedded in the so-called brain adjacent-to-tumor (BAT) region would have the potential to reduce the recurrence rate. PDT is currently being tested clinically for destruction of tumor cells in residual bulk tumor and in the BAT after standard surgery, and for ablation of non-resectable tumors in selected patients. Several available photosensitizers feature excellent tumor–to–normal brain selectivity, but animal studies have shown that damage to normal brain still occurs if the exposure exceeds a certain light fluence (Lilge & Wilson 1998). Furthermore, the selectivity in the BAT can be disrupted by defects in the blood–brain barrier due to transport of the photosensitizer via edema bulk flow (Stummer

et al. 1993). It also remains unclear if tumor cells embedded in the BAT are photosensitized.

PDT is not an established treatment modality for brain tumors. Definite patient benefit has not been confirmed, and the average survival gains reported in pilot studies are only a few months at best. Most clinical trials have been performed with the photosensitizer porfimer sodium and with light delivery to the resection cavity during open surgery. The first placebo controlled *phase III* study was recently finished in Canada and USA (Muller *et al.* 2006). It showed that this treatment does not increase patient survival, at least not with the light doses that were considered safe for porfimer sodium mediated PDT. Hence, other approaches seem necessary to make PDT beneficial for brain tumor patients.

Sensitizers that are more powerful than porfimer sodium and that absorb at longer wavelengths, where light penetrates deeper, have been tested both in animal models and in pilot clinical trials (Lobel *et al.* 2001, Kostron *et al.* 2006). There is also interest in using porphyrin precursor mediated PDT with systemic administration of ALA. Preclinical data from animal models indicate that compared to other sensitizers, ALA photosensitization can lead to a much higher light fluence threshold for necrosis in normal brain tissue (Lilge *et al.* 1996, Olzowy *et al.* 2002). ALA has been tested extensively in Germany for fluorescence-guided resection of brain tumors, and recently a pilot study of ALA mediated PDT has shown that the treatment is safe even for relatively high light doses (Beyer *et al.* 2006). It was also demonstrated that ALA PDT with interstitial light delivery can be used to ablate bulk tumor tissue in inoperable gliomas. However, taking into account the poor results of porfimer sodium PDT it seems unlikely that substantial survival gain can be achieved

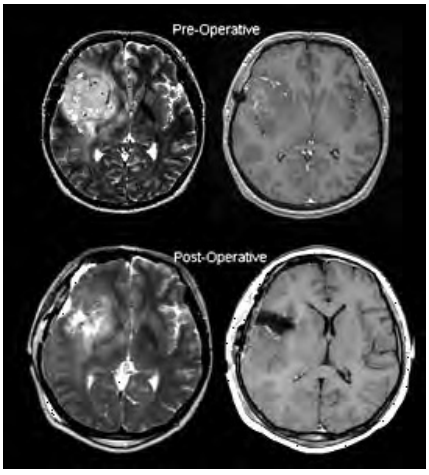

Figure 10: MRI images of a glioblastoma in the right frontal lobe before and after surgery.

from a single treatment session. Madsen *et al.* (2001) have developed an indwelling light applicator (Figure 7c, page 7) designed to be left permanently in the resection cavity after surgery. The intention is then to prevent tumor regrowth by performing repeated PDT sessions throughout the rest of the patient's life. A related approach that is being tested in animal models is to assess other mechanisms of cell death and selectivity by illuminating over several days with ultra-low light dose rates (Bisland *et al.* 2004).

2 Aims of the study

This study investigates the porphyrin precursor mediated photosensitization and the light delivery for PDT of two different indications: non-melanotic skin cancer and malignant brain tumors. The skin studies were a part of developing topical PDT as a standard treatment modality, and were designed to gather detailed knowledge of the accumulation and degradation of porphyrins throughout the drug application and light delivery. For the brain studies, an experimental rat model was used to investigate parameters that are relevant for clinical PDT of brain tumors.

The specific aims of the investigation were:

- To compare the effects of two different light sources on the porphyrins accumulated in skin tumors during topical application of ALA (publication I).
- To describe the distribution of porphyrins in skin lesions and normal skin induced by topical application of MAL-creams (publications II and III).
- To investigate the degree of skin photosensitivity following topical application of MAL (publication III).
- To map the light and temperature distributions during interstitial light delivery to an experimental rat glioma model, and to estimate the optical and thermal properties of brain tissue (publication IV).
- To evaluate to which degree ALA is able to photosensitize glioma tissue and normal brain in a rat model (publications V and VI).
- To evaluate the effect of the rate of light delivery during PDT in a rat glioma model (publication V).

3 Overview of experimental methods

An overview of the methods used in this work is presented below. Further details are available in publications I–VI.

Patients and healthy volunteers (publications I–III)
Patients suffering from AK or BCC were recruited at the outpatient photodynamic clinic at The Norwegian Radium Hospital (I and II). The healthy volunteers (III) were students recruited at the Faculty of Medicine at the University of Oslo.

Animal model (publications IV–VI)
The applied brain tumor model utilized the gliosarcoma cell line BT_4C, which has been established by ethylnitrosourea exposure of transplacental rats of the BDIX inbred strain (Lærum *et al.* 1977). The method for intracranial inoculation of the tumor cells in BDIX rats was refined at Center for Comparative Medicine at Rikshospitalet (Sørensen *et al.* 2002). The *in vivo* experiments were performed at that facility, except from the survival experiments involving tumor-bearing rats in publication VI which were performed at The University of Nevada. Cell cultures of the BT_4C cell line was also used for *in vitro* survival experiments (V and VI).

Drugs and formulations (publications I–III, V, VI)
Skin lesions and normal skin was photosensitized with porphyrin precursors in topical oil-in-water formulations. Two distinct creams were used: an ALA formulation with dimethyl sulfoxide added as a penetration enhancer (I) and the MAL formulation that has become commercially available under the trade name Metvix® (II and III). The creams were covered by an occlusive dressing applied for 3–18 hours. BDIX rats were photosensitized by intraperitoneal (i.p.) injection of a freshly prepared solution of ALA in distilled water (V and VI), or by direct intracranial injection of a buffered ALA solution (VI). In the *in vitro* experiments the BT_4C cells were incubated in 1 or 6 mM ALA in serum-free medium (VI) or 8 mM ALA in medium with 10% calf serum (V). Light exposure and/or pharmacokinetic studies were carried out 1–48 hours following the drug application in the skin studies (I–III), and 4–5 hours following the drug application in the glioma studies (IV–VI).

***In situ* fluorescence measurements (publications I–III)**

Non-invasive point measurements of surface fluorescence were used to monitor the accumulation of photosensitizer in skin and skin lesions. A Perkin Elmer LS-5 spectrofluorimeter was used to deliver excitation light at 405 nm and measure fluorescence at 635 nm via fiberoptic probes. The probe used in the last study (III) resulted in a higher sensitivity for fluorescence detection than the probe used in the two former studies (I and II). Both probes had separate fiber bundles for excitation and fluorescence light directed towards a single sample area. The last study (III) also included measurements using a system based on a CCD spectrometer, which could instantly measure the entire fluorescence spectrum. This allowed for a more accurate background subtraction, resulting in further improvement of the sensitivity for porphyrin fluorescence detection.

Microfluorometry (publications II, V, VI)

The photosensitizer biodistribution in thick nodular BCCs (II) as well as rat brain (V and VI) was studied by fluorescence microscopy of frozen sections. Tissues were cut to 8–10 μm thick sections that were studied under blue excitation light. The sections were then H&E stained for histological identification of tissue types. Image analysis was used to quantify the average fluorescence intensity within a region of interest as a function of the distance to an arbitrary line. This analysis was used to derive depth profiles of the porphyrin distribution in nodular BCCs (II), and to estimate the porphyrin content in glioma and brain tissue as a function of distance to the tumor border (V).

Light exposure (publications I, III–VI)

Skin and skin lesions were irradiated using a light source with a filtered halogen lamp. The system used to irradiate superficial BCCs (in publication I) was a prototype of the CureLight BroadBand® light source used in publication III. The two systems delivered equivalent irradiances of visible light exciting the photosensitizer, but the prototype had more residual output of thermal infrared light. Rat brains and BT_4C glioma cells (IV–VI) were irradiated using lasers with wavelengths within the 635 nm peak of the PpIX excitation spectrum. Most of the animals were irradiated using a diode laser with wavelength 632 nm, where PpIX is excited with at least 90% of the yield at the optimal 635 nm. The light was delivered interstitially from bare 400 μm fiber tips positioned stereotactically. The output power was in the range 4.8–45 mW in the PDT experiments (V and VI) and 15 or 100 mW during measurements of intracranial fluence rates or temperatures (IV).

Measurement and modeling of interstitial light delivery (publications IV, V)
Interstitial probes were assembled and used for stereotactic mapping of intracranial fluence rate and temperature. Publication IV describes how models based either on the diffusion approximation of the transport equation or on Monte Carlo simulation of light propagation were fitted to the measured data. The Monte Carlo model was then used in publication V to estimate the fluence rates and fluences achieved in tumor and brain tissue during interstitial PDT.

Evaluation of treatment effects (publications I, III, V, VI)
The PDT effect in normal skin (III) was evaluated by scoring erythema and pain and by evaluating adverse events such as edema and hyperpigmentation. Also, photobleaching of the photosensitizer during light exposure was measured in superficial BCCs (I) and normal skin (III). The PDT effects on rat glioma and normal brain was evaluated by recording symptom free survival, by observing the morbidity and mortality after light exposure, and by histopathology 15–72 hours post treatment. The histopathology included scoring of tumor necrosis and invasion of neutrophils from H&E stained sections (V and VI), as well as evaluation of edema from sections that were immunostained for plasma protein (V).

4 Synopsis of the results

Publication I:
The bulk of this publication is a clinical trial comparing ALA PDT of superficial BCCs using either 570–740 nm broadband or 630 nm laser light. The trial involved 245 lesions in 83 patients and concluded that 100 J cm^{-2} of laser light and 200 J cm^{-2} of broadband light resulted in the same response rates, cosmetic outcomes, and adverse events. The publication also includes a technical comparison of the two light sources in terms of spectral output and ability to photobleach PpIX. The spectral irradiance of the broadband light source was measured, and calculations of the product integral with the PpIX excitation spectrum suggested that it excited PpIX at 43% of the rate of the laser. Porphyrin fluorescence was measured during light exposure of BCCs. The measurements indicated that the light doses used during the clinical trial were able to photobleach at least 98% of the photosensitizer present in the tumor tissue. There was a tendency to more efficient photobleaching for laser light compared to broad band light, but the data was not sufficient for accurate comparison of the bleaching rates.

Publication II:
The publication presents pharmacokinetic studies investigating the porphyrin inducing effect of MAL creams applied to normal skin, AK, superficial BCC, and thick nodular BCC, varying the cream application time and the MAL concentration.

In vivo surface fluorescence measurements were used to monitor porphyrin accumulation in 18 superficial BCCs, 32 AKs, and adjacent normal skin sites. For both lesion types, the fluorescence increased during the first 13 of 28 hours of continuous MAL application. In normal skin the lowest cream concentration (16 mg g^{-1}) resulted in less fluorescence than the higher concentrations (80 and 160 mg g^{-1}). There were no corresponding statistically significant dependencies in AK or BCC. However, the statistical power to detect MAL concentration dependencies was poor, due to 20-fold lesion-to-lesion variation in the fluorescence intensity. The selectivity between lesions and normal skin was tenfold during the first hours and decreased throughout the application time.

Fluorescence in 32 thick nodular BCCs was studied by analyzing images of frozen tissue sections from biopsies sampled after 3 or 18 hours of MAL application. Fluorescence microscopy images were used to calculate the porphyrin content in

tumor tissue as a function of depth. Within the tumors treated for 3 hours, the average fluorescence intensity depended on the MAL concentration. Increase to 18-hour MAL application significantly enhanced the fluorescence levels in the superficial tumor layers, but not in the deep layers. The fluorescence in epidermis covering the tumor was comparable to that in tumor tissue after 3-hour application, but substantially higher after 18-hour application.

Publication III:
The publication reports a clinical trial evaluating the duration of porphyrin fluorescence and skin photosensitivity after MAL application. Placebo and 160 mg g^{-1} MAL creams were randomly assigned to contralateral sites located at forearms and finger tips of 16 healthy volunteers and applied for 3 hours. Surface fluorescence measurements showed that the porphyrin fluorescence in forearm skin peaked about 1 hour after the cream removal, was halved after 8 hours, and was reduced by more than 90% within 24 hours. Exposure to 50 J cm^{-2} of broadband light 1 and 8 hours following cream removal induced phototoxic reaction in most MAL treated sites. Six subjects also got reactions from exposure at 24 hours following cream removal. At this time, the phototoxicity was positively correlated with residual porphyrin fluorescence. Following cream removal at 48 hours, no photosensitivity or porphyrin fluorescence was detected in any of the subjects. In general, all reactions were mild or moderate, and included pain, erythema, edema, and cases of transient hyperpigmentation. Sites at fingertips showed a 15 times weaker and much slower development of porphyrin fluorescence than the forearm skin, and phototoxic reactions were absent except from sporadic cases of mild pain.

Publication IV:
This publication reports the light and heat distributions during interstitial light delivery to the BDIX/BT_4C rat glioma model used for photodynamic therapy in publications V and VI. Intracranial fluence rates and temperatures were measured with interstitial probes. Mathematical models were then used to derive tissue optical properties and to predict distributions for general tumor and light application geometries. The fluence rates in tumor-free brains agreed well with models based on diffusion theory and Monte Carlo simulation. In both cases the best fit was found for absorption and reduced scattering coefficients of 0.57 and 28 cm^{-1}, respectively. In brains with implanted BT_4C tumors, a discrepancy between the diffusion and Monte Carlo–derived two-layer models was found. The estimates of optical properties for tumor tissue differed, but both models predicted higher absorption and less scattering than in normal brain. Temperatures were measured by inserting thermocouples directly into tumor-free brains. A model based on diffusion theory and the

bioheat equation was found to be in good agreement with the experimental data and predicted a thermal penetration depth of 0.60 cm in normal rat brain.

Publication V:
The publication reports the effect of PDT using systemic ALA and interstitial light delivery to the BDIX/BT_4C glioma model, focusing on the effect of low light delivery rates. *In vitro* experiments showed that the sensitivity of BT_4C multicellular tumor spheroids to ALA-PDT depended on the rate of light delivery (fluence rate). BT_4C tumors were established intracranially in BDIX rats. Microfluorometry of frozen tissue sections showed that photosensitizer was produced with hundredfold tumor-to-normal tissue selectivity after ALA injection, and suggested that 60, 125 and 250 mg kg^{-1} of ALA i.p. resulted in similar porphyrin levels. Four hours after ALA injection (125 mg kg^{-1} i.p.), tumor-bearing animals were treated with 26 J of 632 nm light delivered interstitially over 15 (high fluence rate group) or 90 (low fluence rate group) minutes. Two groups of 7 animals were treated 14 days after tumor induction and were studied by histopathological examination. There was extensive tumor necrosis after low fluence rate PDT, but hardly any necrosis after high fluence rate PDT. Neutrophil infiltration in tumor tissue was increased by PDT, but was similar for both treatment regimens. A low fluence rate PDT applied 9 days after tumor induction resulted in statistically significant prolongation of survival compared to non-treated control animals.

Publication VI:
This publication focuses on the effects of interstitial ALA-PDT on small clusters of BT_4C tumor cells embedded in the normal brain of BDIX rats. Treatment of tumor-free animals demonstrated that ALA-PDT can do significant damage to normal brain if the light fluence is sufficient. Light doses that were safe in the tumor-free animals were used to treat rats 2 days after tumor induction. The treatment failed to prolong the survival compared to untreated controls. In contrast, animals inoculated with tumor cells pre-incubated *in vitro* with ALA showed a significant survival advantage in response to PDT. Consistent with publication V, it was also found that PDT caused significant damage to tumor tissue if the treatment was performed 15 days after the induction. However, histopathology showed that there were viable cells in the tissue invading the normal brain, in spite of the extensive necrosis in gross tumor. Fluorescence microscopy of frozen tissue sections showed that the photosensitizer content was limited and inhomogeneous in the invading tumor tissue. The results show that ALA-PDT could not prevent tumors from forming if the treatment was performed shortly after tumor initiation. This was probably due to insufficient levels of ALA and/or PpIX in the glioma cells.

5 General discussion

5.1 Porphyrin accumulation in normal and tumor tissues: selectivity of the photosensitization

The degree of photosensitization following application of porphyrin precursors depends on the three following steps: (1) Distribution of the porphyrin precursor into the tissue, (2) cellular uptake of the porphyrin precursor and conversion into photosensitive porphyrins, and (3) relocalization, degradation and clearance of porphyrins and porphyrin precursors. The selective photosensitization observed for the two different biological systems studied in this thesis can be due to discrepancies of tumor tissue compared to normal tissue in each of the three steps. The following sections discuss the relevance of each factor for the skin and the brain studies.

For skin tumors, Wennberg *et al.* (2000) have demonstrated that the transport of topical ALA into superficial BCC is much more efficient than into normal skin. It is likely that this is the case also for the porphyrin precursor MAL used in publication II, and that this preferential distribution was a major factor for the observed selectivity of porphyrin fluorescence for BCC and AK (publication II, Figure 2). Another factor contributing to the selectivity is the conversion of MAL into porphyrins. Porphyrins are produced in neoplastic cells and in the epithelial cells of the epidermis, but very little is produced in the dermis (Kennedy *et al.* 1990, Peng *et al.* 2001). The dermis consists mostly of connective tissue rather than cells converting MAL into porphyrins. The porphyrin fluorescence observed from the surface of normal skin (publications II, Figure 2; and III, Figure 1) is from the epidermis, which is thinner—and consequently emit less fluorescence—than the neoplastic tissue in the AKs and BCCs. In addition to this geometrical factor, lesions can have increased fluorescence due to altered activity of the hem synthesis in neoplastic cells (Figure 4, page 4). This effect is seen in several *in vitro* studies reporting higher porphyrin accumulation in tumor cell lines than in normal cell lines. However, the rate of the hem synthesis is known to differ for different tumor types (Collaud *et al.* 2004), and it is not known whether the neoplastic cells in AK and BCC have increased PpIX production compared to the normal epithelial cells in the epidermis. According to the results from nodular BCCs, there seemed to be little selectivity related to increased conversion in the tumor cells. The fluorescence images analyzed in publication II indicate that the epidermis had a porphyrin concentration comparable to that in the underlying tumor (publication II, Figure 4a). Actually, after 18 hours of MAL cream

application (publication II, Figure 4b) there was significantly *more* porphyrin fluorescence in the epidermis than in the underlying tumor.

The shape of the profiles at 18 hours compared to at 3 hours, with the fluorescence decreasing with increasing depth, suggests that the penetration of MAL into the tumors was limited by clearance to the tumor vasculature. Such clearance will not take place in the epidermis since it is not vascularized. Thus, the observed limitation in selectivity for nodular BCCs was probably due to clearance of MAL. Limited selectivity for long application times was the case also for *superficial* lesions (publication II, Figure 3), but this effect might as well be caused by saturation (publication II, Figure 2) of the porphyrin synthesis in the AK and BCC cells combined with slow penetration of MAL into normal epidermis.

For the brain tumor model, the biodistribution studies demonstrated tenfold selectivity versus the BAT (publication V, Figure 4) and hundredfold selectivity versus normal gray matter far from the tumor (publication V, Table 2). It is clear from the literature that for brain tumors the distribution of the porphyrin precursor contributes to selectivity. In general, there is low transfer of ALA into normal brain through the blood–brain barrier. The influx rate has been quantified by Ennis *et al.* (2003). Their results agree with the plasma and brain levels in pharmacokinetic studies employing ^{14}C-labelled ALA and doses relevant for PDT (Hua *et al.* 1995, Obwegeser *et al.* 1998). However, Obwegeser *et al.* found that the ALA concentration ratio between implanted C6 brain tumors and normal brain was merely 4:1. (In the same study the corresponding ratio for the sensitizer m-tetrahydroxyphenylchlorin (Figure 1, page 1) was 80:1.) This suggests that selectivities better than 4:1 are caused by a low conversion into photosensitive porphyrins for the ALA distributed to the normal brain.

As reported in publication V, there was no detectable porphyrin fluorescence in normal gray matter far from the tumor (publication V, Table 2). The same result was found by Hebeda *et al.* (1998) in an extensive biodistribution study involving two rat tumor models. Conversely, Stummer *et al.* (1998) and Lilge *et al.* (1998) reported significant porphyrin levels in normal brain, causing only tenfold tumor selectivity in rat and rabbit models, respectively. The discrepancy in the observed porphyrin levels in normal brain can be due to differences between the implanted tumors. Hebeda *et al.* (1998) and Stummer *et al.* (1998) both showed that the porphyrin content in the normal brain was related to edematous flow from the tumor. Such indirect photosensitization was observed also in the present work, as a resemblance between the porphyrin fluorescence patterns and the edema immunostaining pattern (publication V, Figure 7). Hence, the photosensitization of the normal brain is influenced by the nature of the implanted tumor, and the observed porphyrin level depends on the tumor's ability to photosensitize the brain indirectly.

Nevertheless, the mortality of the tumor-free rats following ALA PDT (publication VI, Figure 4) clearly showed that ALA has a potential for *direct* photosensitization of normal brain. The histopathology (publication VI, Figure 5) and the response to steroid treatment (publication VI, Figure 4) demonstrated that both neuron necrosis and brain edema could have contributed to the mortality. In agreement with our results, ALA PDT effects in tumor-free animals have been reported previously for two different species. For rabbit brain, ALA PDT and porfimer sodium PDT with interstitial light delivery caused similar levels of necrosis in gray matter (Lilge & Wilson 1998). For rat brain, the two drugs have been compared for PDT with external irradiation of the cortex. In that setup, only porfimer sodium caused noticeable necrosis (Olzowy *et al.* 2002), but significant edema occurred in the cortex after ALA PDT (Ito *et al.* 2005).

5.2 Light dosimetry

Although skin lesions and brain tumors are entirely different tumor types, it is of some interest to compare the light dosimetry. The light distribution in the rat model was described in terms of fluences and fluence rates (publications IV and V)[1], whereas in the skin studies (publications I–III), it was simply specified as the exposure of the tissue surfaces. To compare the light dosimetries, the fluence rates in the skin must be estimated. Figure 11 shows the light distributions that can be expected during PDT of skin, BCC, and AK. The calculation was based on a multi layer skin model were the optical properties are estimated from assumed levels of melanin and

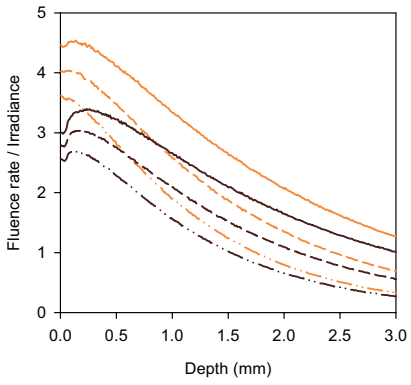

Figure 11: Calculated distributions of 635 nm light in skin with varying melanin and blood content. The graphs show fluence rate relatively to the irradiance as a function of depth, for skin with 1.3% (—) or 6.3% (—) melanin in the epidermis (a range that covers light Caucasian skin (Dwyer *et al.* 2002)), and 0.2% (–) 2% (– –) or 5 % (–·–) blood in the dermis. The calculations were made with a public domain Monte Carlo software (Wang *et al.* 1995). Skin was modeled as a 60 µm epidermal layer covering a dermis with equal amounts of oxygenated and deoxygenated hemoglobin. Optical properties for wavelength 635 nm were derived from spectra published online by Jacques *et al.* (1998).

[1] The results from publication IV were used in publication V, but with preliminary estimates of optical properties that resulted in slightly decreased estimates for fluence rates and fluences. However, the discrepancy was only 10%.

hemoglobin. The blood volume fraction in normal skin is about 0.2% on the average through the dermis, and about 2–5% in the most vascularized layers (Jacques 1996). This range is covered by the calculations of Figure 11. BCCs and AKs have been reported to have a 5–10-fold increased blood content compared to the average in normal skin (i.e. 5–10 × 0.2%) (Newell *et al.* 2003), which also is within the range used in the calculations.

Spectral characteristics of the light also play a major in the dosimetry. As explained in publication I (page 725, "*Calculation of the relative phototherapeutic efficiency*"*)*, more broadband light than monochromatic light must be applied to achieve a certain PDT effect. According to the product integral of the action spectrum for ALA PDT and the lamp emission spectrum, the broadband light was 40% as efficient as 632 nm monochromatic light. To calculate this factor, PpIX's fluorescence excitation spectrum was (as in publication I) used to approximate the action spectrum. The factor is taken into account in Table 1, which estimates the fluences and fluence rates inside skin and skin lesions, based on the distributions in Figure 11. Estimates are made for 50 and 200 J cm^{-2}, the exposure used in publications I and III; 100 J cm^{-2}, the exposure that resulted in 99% photobleaching of surface fluorescence in publication I; and for 75 J cm^{-2}, an exposure proven sufficient for successful PDT of AK and BCC in several clinical trials (Foley 2003).

When comparing the data in Table 1 to the estimates calculated in publication V, the fluences in skin and skin lesions are comparable to, or lower than, the fluences delivered to the brain tumors (publication V, Table 1). Accordingly, the fluence *rates* are comparable to, or higher than, the fluence rates in the brain tumors of the 'Low fluence rate' group. Hence, PDT with the light delivery that was sufficient to photo-

Table 1: Minimum fluences and fluence rates in skin and skin lesions for different light exposures and depths.

Site	Exposure of surface*		Depth	Dosimetry inside tissue†	
	Dose ($J\ cm^{-2}$)	Irradiance ($mW\ cm^{-2}$)	(mm)	Fluence ($J\ cm^{-2}$)	Fluence rate ($mW\ cm^{-2}$)
Normal skin	50	150	0.1‡	50–90	150–270
BCC	100	180	0.1	100–160	180–290
BCC	200	180	1.0§	140–220	130–190
BCC or AK	75	180	0.5	80–110	190–250
BCC or AK	75	180	1.0	60–80	140–190

*Values for the applied broadband light source.

†Corrected with a factor 0.40 to represent values equivalent to 632 nm laser light.

‡Approximately the thickness of the epidermis.

§Maximum thickness of lesions in publication I.

bleach most surface fluorescence in skin (publications I and III), and to successfully treat BCCs and AKs, was insufficient to treat the rat brain tumors (publication V). The PDT could only increase the survival for tumor-bearing rats by a few days (publication V, Figure 8), although the tumors in that experiment were relatively small and received fluences of at least 200 J cm^{-2}. This indicates that systemic ALA application did not photosensitize brain tumors to the same degree that topical ALA or MAL is able to photosensitize skin lesions. Publication VI shows that the porphyrin accumulation was limited and variable in tumor tissue invading the normal brain. This limitation, possibly in combination with insufficient light exposure, may have prevented a complete cytoreduction (publication V, Figure 6a). A similar incomplete cytoreduction was observed by Olzowy *et al.* (2002), who found a non-homogenous distribution of phototoxic damage after ALA PDT in the C6 rat glioma model. The non-homogenous damage suggests that inhomogeneous photosensitizer and/or oxygen supply, rather than insufficient fluence, was the major limitation. However, this was not verified, as the severe side effects of PDT prevented experiments that might have ruled out insufficient fluence as a limiting factor for ALA PDT in the rat model.

5.3 Drug and light delivery for PDT of skin lesions

The approved dose regimen for MAL PDT is application of 160 mg g^{-1} cream for 3 hours, followed by exposure to 75 J cm^{-2} of broadband light (or the equivalent 37 J cm^{-2} of 610–660 nm LED light (Juzeniene *et al.* 2004)). This recommendation is mainly based on evidence from studies of response rates (Foley 2003), but also on the results from the clinical trials described in publications I and II. The advantages of using the 160 mg g^{-1} MAL cream and a 3-hour drug–light interval are thoroughly discussed in publication II. For this drug dosage, the porphyrin fluorescence was selective for the tumor tissue and was distributed more than a millimeter into thick BCCs. The high response rates found in several studies demonstrate that sufficient photosensitization of AK and BCC lesions is achieved (Foley 2003). Furthermore, the results in publication III demonstrate that the photosensitization of normal skin is too low to cause severe damage. Selectivity does not appear to be a limiting factor for MAL PDT in the skin when 160 mg g^{-1} MAL cream is applied for 3 hours.

In some cases, a BCC or AK lesion does not respond well to MAL PDT. Two treatment cycles are often required to achieve good results (Braathen *et al.* 2007). Two-year accumulated treatment failure rates of around 25% have been reported for so-called "difficult to treat" nodular BCCs (Horn *et al.* 2003, Vinciullo *et al.* 2005), and the 3-year recurrence rate for nodular BCCs is around 15% (for thick debulked tumors) (Soler *et al.* 2001). The cases of treatment failure might be due to incomplete photosensitization of the neoplastic tissue. The fluorescence depth profiles from

nodular BCCs (publication II, Figure 4a) show an intensity decrease below 1.0–1.2 mm. The intensity also varied considerably from lesion to lesion, and in some cases there was considerable local variation across a single lesion (results not shown). The tumors in publication II were not debulked. Debulking is necessary to treat thick nodular BCCs (Warloe 1995), probably because it improves the penetration of MAL into the tumor tissue. This suggests that the observed variation in the fluorescence in the thick BCCs was due to insufficient availability of MAL. However, insufficient photosensitization can in general also be due to poor porphyrin synthesis in the tumor cells. Morgan *et al.* (2004) have showed that the cellular expression of the peripheral benzodiazepine receptor—a crucial component in the heme synthesis (Figure 4, page 4)— can be heterogeneous in BCCs, particularly for subtypes that respond poorly to PDT. According to their results, some tumors may respond poorly PDT, even if there is adequate penetration of both the porphyrin precursor and the light.

The doses of red light used to successfully treat BCC have been in the range of 50–200 J cm^{-2}, delivered at irradiances in the range of 100–200 mW cm^{-2} (Szeimies *et al.* 2005, Garcia-Zuazaga *et al.* 2005). Both laser and broadband sources have been used. In the clinical trial of publication I, the ALA treated superficial BCCs were exposed to 200 J cm^{-2} of broadband light or 100 J cm^{-2} of laser light. Less light was used in publication III, where the MAL treated normal skin sites were exposed to 50 J cm^{-2} of broadband light. In both cases, the fluorescence measurements and the expected depth of the PpIX biodistribution indicated that at least 98% of the available photosensitizer was photobleached during the light exposures. This almost complete photobleaching of the sensitizer can explain why the different light sources had the same therapeutic effect.

However, there is not a general direct relationship between PDT-effect and photobleaching (Robinson *et al.* 1999). Particularly in the case of hypoxia, it has been demonstrated that PpIX is photobleached independently of the PDT (Dysart & Patterson 2006). The tissue oxygenation and the PDT effect can be reduced if the fluence rate is too high, i.e., if the applied irradiance is too high (Foster *et al.* 1991). In publication I, the irradiance of the laser light was 120–150 mW cm^{-2}. Taking into account the differences in the emission spectra (publication I, page 725, "*Calculation of the relative phototherapeutic efficiency*"), the effective irradiance of the broadband light was 43–77 mW cm^{-2}. Hence, in spite of the equal degree of photobleaching, it is possible that more $^1O_2$ was produced during exposure to broadband light than to laser light. Still, no differences between the light sources were seen in the clinical trial reported in publication I. This either means that the two light exposure alternatives were equivalent with respect to the PDT effect, or that the light exposure was not a limiting factor.

5.4 Effects, limitations, and clinical relevance of PDT in the glioma model

The experiments indicated that ALA PDT may have a potential to treat glioma. However, considerable limitations related to light distribution, side effects, and drug distribution were also found. The light distribution is discussed in publication IV. The penetration depth (δ) in normal rat brain was found to be 1.4 mm, the same as previously reported for human brain (Muller & Wilson 1986). The tumor had δ around 1.7 mm, which is less than the 2.4–3.8 mm reported for human tumors and BAT (Muller & Wilson 1986, Beck *et al.* 2003). According to the calculations in publication IV, the limited light penetration in BT_4C tumors was due to strong light absorption ($\mu_{a,tumor} = 1.4\ cm^{-1}$). The high $\mu_{a,tumor}$ was not unexpected, as BT_4C tumor tissue is well vascularized (Sørensen *et al.* 2002) resulting in enhanced light absorption by hemoglobin. During PDT, when the tumor contains light absorbent photosensitizer, $\mu_{a,tumor}$ is likely to be further increased.

The high light attenuation and the use of an implanted point source resulted in strong spatial gradients in the delivered fluences (publications IV, Figure 4; and V, Table 1). In patients, the larger light penetration depth and the use of non–point source applicators would result in smaller gradients. Still, the fraction of the brain exposed during PDT in rats will generally be much larger than in the human brain. Furthermore, the small size and limited buffering volume of the rats' intracranial space quickly leads to increased pressure following PDT, as a result of cerebral edema and normal tumor progression. These differences between small animals and humans are important for the interpretation of the observed mortality and morbidity (publications V and VI). Recently, no severe side effects were observed among 41 patients participating in a pilot study of ALA PDT for malignant glioma (Beyer *et al.* 2006). The study included both intraoperative treatments and minimally invasive interstitial treatments. In both cases, the BAT was exposed to fluences at least 5 times higher than for the rats in publication V (W. Beyer, personal communication). This strongly suggests that the severity of the side effects observed in the rat model is not clinically relevant.

It was evident that the ALA PDT was not effective if the light was delivered at too high fluence rates, and this is discussed in detail in publication V. At lower fluence rates the treatment was effective for gross tumor, but (as explained in publication VI) it failed to treat the invading part of the tumor. Treatment of gross tumor can be valuable for inoperable patients. However, in most cases of malignant glioma the core is necrotic, and the tumor growth mainly takes place in the border region (Preusser *et al.* 2006). The prime target for the PDT is thus the tumor border region and the BAT. As described above, the fluences in the rat model were limited by side effects that are not necessarily clinically relevant. It is possible that higher fluences

could have inactivated the tumor border more completely. The porphyrin fluorescence was inhomogeneous, but even the areas with the lowest fluorescence had similar fluorescence as the BAT (publication VI, Figure 3), and this level (publication V, Figure 4) may have been sufficient to inactivate tumor cells if the light fluence had been sufficient. However, even when the treatment was preformed 2 days after the tumor cell inoculation (publication VI, Figure 7), ALA PDT failed to prolong the survival. In these cases the cells were positioned 1–2 mm from the fiber tip, and should have received high fluences. Hence, the small newly implanted BT_4C tumors embedded in normal brain were not significantly photosensitized by the ALA distribution. This experiment needs to be repeated with other tumor models to investigate to which degree insufficient photosensitization is a general phenomenon for systemic ALA distribution to brain tumors.

6 Conclusions

- Both 200 J cm^{-2} of red broadband light and 100 J cm^{-2} of 630 nm laser light was sufficient to photobleach the porphyrins formed in superficial BCC after topical ALA application to below 2%. This can explain why PDT with the two light sources resulted in the same response rates and cosmetic outcomes.
- The porphyrin fluorescence intensity in the surface of BCCs and AKs increased, and the lesion to normal skin selectivity ratio decreased, during the first 13–20 hours of continuous MAL application. However, application for 3 and 18 hours resulted in similar porphyrin fluorescence levels in the deeper layers of thick nodular BCCs. Only for the 3-hour application the fluorescence depended significantly on the MAL concentration in the cream.
- Topical MAL application for 3 hours followed by exposure to red light induced mild or moderate phototoxicity in normal skin. The photosensitivity ceased within 24–48 hours after cream removal, and its duration was associated with the degradation of porphyrins.

- In the rat glioma model, intracranial fluence rates and temperatures measured during interstitial light delivery corresponded well to mathematical models of light propagation. The models were used to estimate the fluence rate and temperature distributions occurring during PDT. An absorption coefficient of 0.57 cm^{-1}, a reduced scattering coefficient of 28 cm^{-1}, and a thermal penetration depth of 0.60 cm was estimated for the rat brain.
- Reduction of the light delivery rate strongly enhanced the effect of interstitial ALA PDT on glioma tissue.
- ALA PDT caused significant necrosis in gross glioma tissue. However, the cytoreduction was not complete, and only minor tumor growth delay was achieved. The light dose usable in the experiments was limited by severe side effects that were probably related to the small size of the rat brain.
- The ALA application induced highly selective porphyrin production in glioma tissue, but the porphyrin levels in the region of the tumor invading the normal brain were inhomogeneous and limited. Systemic ALA distribution did not photosensitize microclusters of newly implanted glioma cells.

7 Future prospects

7.1 Optimization of MAL PDT for skin lesions

There are no published clinical studies comparing the efficacy of different light doses for MAL PDT of skin lesions. For MAL PDT, the multicenter studies leading to approval for AK and BCC have all been performed with 75 J cm^{-2} of broadband light (or 37 J cm^{-2} of 610–660 nm LED light). At the Norwegian Radium Hospital, successful MAL PDT of superficial BCC has been achieved using just 50 J cm^{-2} of broadband light (Warloe *et al.*, personal communication[1]). A clinical trial comparing different light doses could establish the minimum light dose necessary for MAL PDT for different lesion types. The primary end point in such a study would be treatment response rates, but analysis of light penetration and photobleaching would be of interest when interpreting the data.

At present, an LED light source (Aktilite®, PhotoCure ASA, Norway) with an irradiance of 75–90 mW cm^{-2} is by far the most common light source for MAL PDT. Light sources with higher irradiances could be assembled using LEDs with a higher output power. Up to 200 mW cm^{-2} can be applied to skin without causing hyperthermia. At this irradiance the 37 J cm^{-2} can be delivered in just three minutes. However, it would be necessary to perform a clinical trial investigating if the increased irradiance affects the treatment response and the pain felt during the light exposure.

In some cases, a thick BCC is too dense to allow the debulking that is considered essential for successful MAL PDT. At The Norwegian Radium Hospital such lesions are prepared by making perforations through the surface with a cannula or needle. This is thought to enhance the penetration of MAL, but the effect of the procedure has not been documented. A biodistribution study, using the same methods as in publication II, would demonstrate whether the perforation procedure does enhance the porphyrin accumulation in the tumor tissue.

[1] Complete response of 90%, sustained for at least 5 years in 359 of 397 superficial BCCs after a single MAL PDT.

7.2 PDT of brain tumors

Further experiments using the BT_4C rat glioma model that would be of interest include: To quantitatively measure the amount of PpIX formed in normal brain using chemical extraction of porphyrins; to investigate whether the increased survival observed for low fluence rate PDT (publication V, Figure 8) can be enhanced by increasing the light dose; to increase the steroid dose in attempt to reduce side effects related to brain edema; and to find out if ALA PDT kills glioma cells by apoptosis, and whether this depends on the fluence rate. The observation that newly implanted BT_4C cells are not photosensitized by systemic ALA distribution may indicate a critical limitation of ALA PDT, and should be tested using other animal glioma models.

If complete cytoreduction can not be achieved by a single ALA PDT session, the treatment modality may still be effective by using multiple treatments. This strategy has proven useful for topical MAL PDT of skin cancer, where tumors responding poorly to a single treatment often are cured by multiple treatments (Braathen *et al.* 2007). Pilot experiments with the BT_4C model indicated that the observed side effects of ALA PDT can be a major obstacle for testing repetitive PDT in rats (unpublished results). Furthermore, the model features a life span from inoculation to lethal tumor of only 20 days. A more realistic, slow growing tumor model would be more suitable to test clinically relevant repetitive PDT schedules. Alternatively, repetitive ALA PDT could be tested directly in patients. If (single treatment) intracranial ALA PDT is proven safe by the ongoing clinical trial in Munich (Beyer *et al.* 2006), one way to enhance the treatment would be to perform repeated treatments.

References

Ackroyd R., Kelty C., Brown N., and Reed M. (2001): The history of photodetection and photodynamic therapy. *Photochem.Photobiol.* **74**: 656–669.

Beck T.J., Beyer W., Pongratz T., Stummer W., Waidelich R.M., Stepp H., Wagner S., and Baumgartner R. (2003): Clinical determination of tissue optical properties in vivo by spatially resolved reflectance measurements. *Proceedings of SPIE* **5138**: 96–105.

Berg K., Selbo P.K., Weyergang A., Dietze A., Prasmickaite L., Bonsted A., Engesæter B.O., Angell-Petersen E., Warloe T., Frandsen N., and Høgset A. (2005): Porphyrin-related photosensitizers for cancer imaging and therapeutic applications. *J.Microsc.* **218**: 133–147.

Beyer W., Beck T.J., Stepp H., Tonn J.C., Reulen H.J., Kreth F., and Stummer W. (2006): Munich experiences with 5-ALA-PDT of malignant gliomas. *6th International Symposium on Photodynamic Diagnosis and Therapy in Clinical Practice, October 10–14*, (Abstract).

Bigelow C.E., Mitra S., Knuechel R., and Foster T.H. (2001): ALA- and ALA-hexylester-induced protoporphyrin IX fluorescence and distribution in multicell tumour spheroids. *Br.J.Cancer* **85**: 727–734.

Bisland S.K., Lilge L., Lin A., Rusnov R., and Wilson B.C. (2004): Metronomic photodynamic therapy as a new paradigm for photodynamic therapy: rationale and preclinical evaluation of technical feasibility for treating malignant brain tumors. *Photochem.Photobiol.* **80**: 22–30.

Blum H.F. (1941): *Photodynamic action and diseases caused by light*. Reinhold Publishing Corporation, New York.

Braathen L.R., Szeimies R.M., Basset-Seguin N., Bissonnette R., Foley P., Pariser D., Roelandts R., Wennberg A.M., and Morton C.A. (2007): Guidelines on the use of photodynamic therapy for nonmelanoma skin cancer: an international consensus. *J.Am.Acad.Dermatol.* **56**: 125–143.

Brancaleon L. and Moseley H. (2002): Laser and non-laser light sources for photodynamic therapy. *Lasers Med.Sci.* **17**: 173–186.

Brooke R.C. (2005): Basal cell carcinoma. *Clin.Med.* **5**: 551–554.

Casas A., Perotti C., Fukuda H., Rogers L., Butler A.R., and Batlle A. (2001): ALA and ALA hexyl ester-induced porphyrin synthesis in chemically induced skin tumours: the role of different vehicles on improving photosensitization. *Br.J.Cancer* **85**: 1794–1800.

CBTRUS (2005): *Statistical Report: Primary Brain Tumors in the United States, 1998–2002*. Central Brain Tumor Registry of the United States, USA.

Chen B., Pogue B.W., Hoopes P.J., and Hasan T. (2006): Vascular and cellular targeting for photodynamic therapy. *Crit Rev.Eukaryot.Gene Expr.* **16**: 279–305.

Cheong W.F. (1995): Summary of optical properties. In: *Optical-Thermal Response of Laser-Irradiated Tissue*, edited by A.J.Welch and M.J.van Gemert, pp. 275–303. Plenum Press, New York.

Chopp M., Madigan L., Dereski M., Jiang F., and Li Y. (1996): Photodynamic therapy of human glioma (U87) in the nude rat. *Photochem.Photobiol.* **64**: 707–711.

Collaud S., Juzeniene A., Moan J., and Lange N. (2004): On the selectivity of 5-aminolevulinic acid–induced protoporphyrin IX formation. *Curr.Med.Chem.Anti.-Canc.Agents* **4**: 301–316.

de Rosa F.S. and Bentley M.V. (2000): Photodynamic therapy of skin cancers: sensitizers, clinical studies and future directives. *Pharm.Res.* **17**: 1447–1455.

Dougherty T.J., Gomer C.J., Henderson B.W., Jori G., Kessel D., Korbelik M., Moan J., and Peng Q. (1998): Photodynamic therapy. *J.Natl.Cancer Inst.* **90**: 889–905.

Driver I., Lowdell C.P., and Ash D.V. (1991): In vivo measurement of the optical interaction coefficients of human tumours at 630 nm. *Phys.Med Biol.* **36**: 805–813.

Dwyer T., Blizzard L., Ashbolt R., Plumb J., Berwick M., and Stankovich J.M. (2002): Cutaneous melanin density of Caucasians measured by spectrophotometry and risk of malignant melanoma, basal cell carcinoma, and squamous cell carcinoma of the skin. *Am.J.Epidemiol.* **155**: 614–621.

Dysart J.S. and Patterson M.S. (2006): Photobleaching kinetics, photoproduct formation, and dose estimation during ALA induced PpIX PDT of MLL cells under well oxygenated and hypoxic conditions. *Photochem.Photobiol.Sci.* **5**: 73–81.

Einstein A. (1912): Thermodynamische Begründung des photochemischen Äquivalentgesetzes. *Annalen der Physik* **37**: 832–838.

Ennis S.R., Novotny A., Xiang J., Shakui P., Masada T., Stummer W., Smith D.E., and Keep R.F. (2003): Transport of 5-aminolevulinic acid between blood and brain. *Brain Res.* **959**: 226–234.

Foley P. (2003): Clinical efficacy of methyl aminolevulinate (Metvix) photodynamic therapy. *J.Dermatolog.Treat.* **14 Suppl 3**: 15–22.

Foster T.H., Murant R.S., Bryant R.G., Knox R.S., Gibson S.L., and Hilf R. (1991): Oxygen consumption and diffusion effects in photodynamic therapy. *Radiat.Res.* **126**: 296–303.

Fritsch C., Homey B., Stahl W., Lehmann P., Ruzicka T., and Sies H. (1998): Preferential relative porphyrin enrichment in solar keratoses upon topical application of delta-aminolevulinic acid methylester. *Photochem.Photobiol.* **68**: 218–221.

Fuchs J. and Thiele J. (1998): The role of oxygen in cutaneous photodynamic therapy. *Free Radic.Biol.Med.* **24**: 835–847.

Fukuda H., Paredes S., and Batlle A.M. (1992): Tumour-localizing properties of porphyrins. In vivo studies using free and liposome encapsulated aminolevulinic acid. *Comp.Biochem.Physiol.B* **102**: 433–436.

Garcia-Zuazaga J., Cooper K.D., and Baron E.D. (2005): Photodynamic therapy in dermatology: current concepts in the treatment of skin cancer. *Expert.Rev.Anticancer Ther.* **5**: 791–800.

Gaullier J.M., Berg K., Peng Q., Anholt H., Selbo P.K., Ma L.W., and Moan J. (1997): Use of 5-aminolevulinic acid esters to improve photodynamic therapy on cells in culture. *Cancer Res.* **57**: 1481–1486.

Gederaas O.A., Holroyd A., Brown S.B., Vernon D., Moan J., and Berg K. (2001): 5-Aminolaevulinic acid methyl ester transport on amino acid carriers in a human colon adenocarcinoma cell line. *Photochem.Photobiol.* **73**: 164–169.

Hebeda K.M., Saarnak A.E., Olivo M., Sterenborg H.J., and Wolbers J.G. (1998): 5-Aminolevulinic acid induced endogenous porphyrin fluorescence in 9L and C6 brain tumours and in the normal rat brain. *Acta Neurochir.(Wien)* **140**: 503–512.

Hopper C., Kubler A., Lewis H., Tan I.B., and Putnam G. (2004): mTHPC-mediated photodynamic therapy for early oral squamous cell carcinoma. *Int.J.Cancer* **111**: 138–146.

Horn M., Wolf P., Wulf H.C., Warloe T., Fritsch C., Rhodes L.E., Kaufmann R., de Rie M., Legat F.J., Stender I.M., Soler A.M., Wennberg A.M., Wong G.A., and Larkö O. (2003): Topical methyl aminolaevulinate photodynamic therapy in patients with basal cell carcinoma prone to complications and poor cosmetic outcome with conventional treatment. *Br.J.Dermatol.* **149**: 1242–1249.

Hua Z., Gibson S.L., Foster T.H., and Hilf R. (1995): Effectiveness of delta-aminolevulinic acid-induced protoporphyrin as a photosensitizer for photodynamic therapy in vivo. *Cancer Res.* **55**: 1723–1731.

Huld T. and Suri M. (2006): PVGIS Solar Irradiance Data Utility. *http://re.jrc.ec.europa.eu/pvgis/sunraddayframe.php*, PVGIS: Geographical Assessment of Solar Energy Resource and Photovoltaic Technology, European Commission, DG - Joint Research Centre, Institute for Environment and Sustainability, Ispra, Italy.

ICNIRP (1997): Guidelines on limits of exposure to broad-band incoherent optical radiation (0.38 to 3 microM). International Commission on Non-Ionizing Radiation Protection. *Health Phys.* **73**: 539–554.

Ito S., Rachinger W., Stepp H., Reulen H.J., and Stummer W. (2005): Oedema formation in experimental photo-irradiation therapy of brain tumours using 5-ALA. *Acta Neurochir.(Wien.)* **147**: 57–65.

Jacques S.L. (1996): Origins of tissue optical properties in the UVA, Visible, and NIR regions. In: *OSA Topics in Optics and Photonics, Advances in Optical Imaging and Photon Migration*, edited by R.R.Alfano and J.G.Fujimoto, pp. 364–369. Optical Society of America, Washington DC.

Jacques S.L. (1998): Skin Optics Summary. *http://omlc.ogi.edu/news/jan98/skinoptics.html*, Oregon Medical Laser Center, Providence St.Vincent Medical Center, USA.

Jeffes E.W. (2002): Levulan: the first approved topical photosensitizer for the treatment of actinic keratosis. *J.Dermatolog.Treat.* **13 Suppl 1**: S19–S23.

Jocham D., Witjes F., Wagner S., Zeylemaker B., van M.J., Grimm M.O., Muschter R., Popken G., Konig F., Knuchel R., and Kurth K.H. (2005): Improved detection and treatment of bladder cancer using hexaminolevulinate imaging: a prospective, phase III multicenter study. *J.Urol.* **174**: 862–866.

Juzeniene A., Juzenas P., Ma L.W., Iani V., and Moan J. (2004): Effectiveness of different light sources for 5-aminolevulinic acid photodynamic therapy. *Lasers Med.Sci.* **19**: 139–149.

Kasche A., Luderschmidt S., Ring J., and Hein R. (2006): Photodynamic therapy induces less pain in patients treated with methyl aminolevulinate compared to aminolevulinic acid. *J.Drugs Dermatol.* **5**: 353–356.

Kawaoka K., Khan A.U., and Kearns D.R. (1967): Role of Singlet Excited States of Molecular Oxygen in the Quenching of Organic Triplet States. *J.Chem.Phys.* **46**: 1842–1853.

Kennedy J.C., Pottier R.H., and Pross D.C. (1990): Photodynamic therapy with endogenous protoporphyrin IX: basic principles and present clinical experience. *J Photochem Photobiol B* **6**: 143–148.

Kloek J. and Beijersbergen v.H. (1996): Prodrugs of 5-aminolevulinic acid for photodynamic therapy. *Photochem.Photobiol.* **64**: 994–1000.

Kostron H., Fiegele T.H., and Akatuna E. (2006): Photodynamic diagnosis and photodynamic therapy for recurrent glioblastomas – How to Improve the results? *6th International Symposium on Photodynamic Diagnosis and Therapy in Clinical Practice, October 10–14*, (Abstract).

Kreftregisteret (2006): *Cancer in Norway 2004*. Cancer Registry of Norway, Institute of Population-based Cancer Research, Norway.

Lærum O.D., Rajewsky M.F., Schachner M., Stavrou D., Haglid K.G., and Haugen A. (1977): Phenotypic properties of neoplastic cell lines developed from fetal rat brain cells in culture after exposure to ethylnitrosourea in vivo. *Z.Krebsforsch.Klin.Onkol.Cancer Res.Clin.Oncol.* **89**: 273–295.

Lange N., Jichlinski P., Zellweger M., Forrer M., Marti A., Guillou L., Kucera P., Wagnieres G., and van den Bergh H. (1999): Photodetection of early human bladder cancer based on the fluorescence of 5-aminolaevulinic acid hexylester-induced protoporphyrin IX: a pilot study. *Br.J.Cancer* **80**: 185–193.

Lilge L., Olivo M.C., Schatz S.W., MaGuire J.A., Patterson M.S., and Wilson B.C. (1996): The sensitivity of normal brain and intracranially implanted VX2 tumour to interstitial photodynamic therapy. *Br.J.Cancer* **73**: 332–343.

Lilge L. and Wilson B.C. (1998): Photodynamic therapy of intracranial tissues: a preclinical comparative study of four different photosensitizers. *J.Clin.Laser Med.Surg.* **16**: 81–91.

Lo J.S., Snow S.N., Reizner G.T., Mohs F.E., Larson P.O., and Hruza G.J. (1991): Metastatic basal cell carcinoma: report of twelve cases with a review of the literature. *J.Am.Acad.Dermatol.* **24**: 715–719.

Lobel J., MacDonald I.J., Ciesielski M.J., Barone T., Potter W.R., Pollina J., Plunkett R.J., Fenstermaker R.A., and Dougherty T.J. (2001): 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) in a nude rat glioma model: implications for photodynamic therapy. *Lasers Surg.Med.* **29**: 397–405.

Lopez R.F., Lange N., Guy R., and Bentley M.V. (2004): Photodynamic therapy of skin cancer: controlled drug delivery of 5-ALA and its esters. *Adv.Drug Deliv.Rev.* **56**: 77–94.

Lui H., Hobbs L., Tope W.D., Lee P.K., Elmets C., Provost N., Chan A., Neyndorff H., Su X.Y., Jain H., Hamzavi I., McLean D., and Bissonnette R. (2004): Photodynamic therapy of multiple nonmelanoma skin cancers with verteporfin and red light-emitting diodes: two-year results evaluating tumor response and cosmetic outcomes. *Arch.Dermatol.* **140**: 26–32.

Madsen S.J., Sun C.H., Tromberg B.J., and Hirschberg H. (2001): Development of a novel indwelling balloon applicator for optimizing light delivery in photodynamic therapy. *Lasers Surg.Med.* **29**: 406–412.

Malik Z. and Lugaci H. (1987): Destruction of erythroleukaemic cells by photoactivation of endogenous porphyrins. *Br.J.Cancer* **56**: 589–595.

Martin A., Tope W.D., Grevelink J.M., Starr J.C., Fewkes J.L., Flotte T.J., Deutsch T.F., and Anderson R.R. (1995): Lack of selectivity of protoporphyrin IX fluorescence for basal cell carcinoma after topical application of 5-aminolevulinic acid: implications for photodynamic treatment. *Arch.Dermatol.Res* **287**: 665–674.

Moan J. and Berg K. (1991): The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. *Photochem.Photobiol.* **53**: 549–553.

Moan J., Ma L.W., and Iani V. (2001): On the pharmacokinetics of topically applied 5-aminolevulinic acid and two of its esters. *Int.J.Cancer* **92**: 139–143.

Moore J.V., West C.M., and Whitehurst C. (1997): The biology of photodynamic therapy. *Phys.Med.Biol.* **42**: 913–935.

Morgan J., Oseroff A.R., and Cheney R.T. (2004): Expression of the peripheral benzodiazepine receptor is decreased in skin cancers in comparison with normal skin. *Br.J.Dermatol.* **151**: 846–856.

Muller P., Wilson B., Lilge L., Bogaars A., Hetzel F., Chen Q., Fullagar T., and Abrams J. (2006): Photodynamic therapy of brain tumors. *6th International Symposium on Photodynamic Diagnosis and Therapy in Clinical Practice, October 10–14*, (Abstract).

Muller P.J. and Wilson B.C. (1986): An update on the penetration depth of 630 nm light in normal and malignant human brain tissue in vivo. *Phys.Med Biol.* **31**: 1295–1297.

Newell B., Bedlow A.J., Cliff S., Drysdale S.B., Stanton A.W., and Mortimer P.S. (2003): Comparison of the microvasculature of basal cell carcinoma and actinic keratosis using intravital microscopy and immunohistochemistry. *Br.J.Dermatol.* **149**: 105–110.

Niedre M., Patterson M.S., and Wilson B.C. (2002): Direct near-infrared luminescence detection of singlet oxygen generated by photodynamic therapy in cells in vitro and tissues in vivo. *Photochem.Photobiol.* **75**: 382–391.

Niedre M.J., Yu C.S., Patterson M.S., and Wilson B.C. (2005): Singlet oxygen luminescence as an in vivo photodynamic therapy dose metric: validation in normal mouse skin with topical amino-levulinic acid. *Br.J.Cancer* **92**: 298–304.

Obwegeser A., Jakober R., and Kostron H. (1998): Uptake and kinetics of 14C-labelled meta-tetrahydroxyphenylchlorin and 5-aminolaevulinic acid in the C6 rat glioma model. *Br.J.Cancer* **78**: 733–738.

Olzowy B., Hundt C.S., Stocker S., Bise K., Reulen H.J., and Stummer W. (2002): Photoirradiation therapy of experimental malignant glioma with 5-aminolevulinic acid. *J.Neurosurg.* **97**: 970–976.

Ortonne J.P. (2002): From actinic keratosis to squamous cell carcinoma. *Br.J.Dermatol.* **146 Suppl 61**: 20–23.

Oseroff A.R., Blumenson L.R., Wilson B.D., Mang T.S., Bellnier D.A., Parsons J.C., Frawley N., Cooper M., Zeitouni N., and Dougherty T.J. (2006): A dose ranging study of photodynamic therapy with porfimer sodium (Photofrin) for treatment of basal cell carcinoma. *Lasers Surg.Med.* **38**: 417–426.

Pech O., Gossner L., May A., Rabenstein T., Vieth M., Stolte M., Berres M., and Ell C. (2005): Long-term results of photodynamic therapy with 5-aminolevulinic acid for superficial Barrett's cancer and high-grade intraepithelial neoplasia. *Gastrointest.Endosc.* **62**: 24–30.

Peng Q., Evensen J.F., Rimington C., and Moan J. (1987): A comparison of different photosensitizing dyes with respect to uptake C3H-tumors and tissues of mice. *Cancer Lett.* **36**: 1–10.

Peng Q., Moan J., Warloe T., Iani V., Steen H.B., Bjørseth A., and Nesland J.M. (1996): Build-up of esterified aminolevulinic-acid-derivative-induced porphyrin fluorescence in normal mouse skin. *J.Photochem.Photobiol.B* **34**: 95–96.

Peng Q., Soler A.M., Warloe T., Nesland J.M., and Giercksky K.E. (2001): Selective distribution of porphyrins in thick basal cell carcinoma after topical application of methyl 5-aminolevulinate. *J.Photochem.Photobiol.B* **62**: 140–145.

Peng Q., Warloe T., Moan J., Heyerdahl H., Steen H.B., Nesland J.M., and Giercksky K.E. (1995): Distribution of 5-aminolevulinic acid-induced porphyrins in noduloulcerative basal cell carcinoma. *Photochem.Photobiol.* **62**: 906–913.

Perotti C., Casas A., Fukuda H., Sacca P., and Batlle A. (2002): ALA and ALA hexyl ester induction of porphyrins after their systemic administration to tumour bearing mice. *Br.J.Cancer* **87**: 790–795.

Perotti C., Fukuda H., DiVenosa G., MacRobert A.J., Batlle A., and Casas A. (2004): Porphyrin synthesis from ALA derivatives for photodynamic therapy. In vitro and in vivo studies. *Br.J.Cancer* **90**: 1660–1665.

Preusser M., Haberler C., and Hainfellner J.A. (2006): Malignant glioma: neuropathology and neurobiology. *Wien.Med.Wochenschr.* **156**: 332–337.

Redmond R.W. and Gamlin J.N. (1999): A compilation of singlet oxygen yields from biologically relevant molecules. *Photochem.Photobiol.* **70**: 391–475.

Robinson D.J., de Bruijn H.S., van der Veen N., Stringer M.R., Brown S.B., and Star W.M. (1999): Protoporphyrin IX fluorescence photobleaching during ALA-mediated photodynamic therapy of UVB-induced tumors in hairless mouse skin. *Photochem.Photobiol.* **69**: 61–70.

Rud E., Gederaas O., Høgset A., and Berg K. (2000): 5-aminolevulinic acid, but not 5-aminolevulinic acid esters, is transported into adenocarcinoma cells by system BETA transporters. *Photochem.Photobiol.* **71**: 640–647.

Soler A.M., Warloe T., Berner A., and Gierckskу K.E. (2001): A follow-up study of recurrence and cosmesis in completely responding superficial and nodular basal cell carcinomas treated with methyl 5-aminolaevulinate-based photodynamic therapy alone and with prior curettage. *Br.J.Dermatol.* **145**: 467–471.

Soler A.M., Warloe T., Tausjø J., and Berner A. (1999): Photodynamic therapy by topical aminolevulinic acid, dimethylsulphoxide and curettage in nodular basal cell carcinoma: a one-year follow-up study. *Acta Derm.Venereol.* **79**: 204–206.

Sørensen D.R., Read T.A., Porwol T., Olsen B.R., Timpl R., Sasaki T., Iversen P.O., Benestad H.B., Sim B.K., and Bjerkvig R. (2002): Endostatin reduces vascularization, blood flow, and growth in a rat gliosarcoma. *Neuro.-oncol.* **4**: 1–8.

Star W.M. (1997): Light dosimetry in vivo. *Phys.Med.Biol.* **42**: 763–787.

Stummer W., Gotz C., Hassan A., Heimann A., and Kempski O. (1993): Kinetics of Photofrin II in perifocal brain edema. *Neurosurg.* **33**: 1075–1081.

Stummer W., Pichlmeier U., Meinel T., Wiestler O.D., Zanella F., and Reulen H.J. (2006): Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol.* **7**: 392–401.

Stummer W., Stocker S., Novotny A., Heimann A., Sauer O., Kempski O., Plesnila N., Wietzorrek J., and Reulen H.J. (1998): In vitro and in vivo porphyrin accumulation by C6 glioma cells after exposure to 5-aminolevulinic acid. *J.Photochem.Photobiol.B* **45**: 160–169.

Stupp R., Mason W.P., van den Bent M.J., Weller M., Fisher B., Taphoorn M.J., Belanger K., Brandes A.A., Marosi C., Bogdahn U., Curschmann J., Janzer R.C., Ludwin S.K., Gorlia T., Allgeier A., Lacombe D., Cairncross J.G., Eisenhauer E., and Mirimanoff R.O. (2005): Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N.Engl.J.Med.* **352**: 987–996.

Szeimies R.M., Karrer S., Radakovic-Fijan S., Tanew A., Calzavara-Pinton P.G., Zane C., Sidoroff A., Hempel M., Ulrich J., Proebstle T., Meffert H., Mulder M., Salomon D., Dittmar H.C., Bauer J.W., Kernland K., and Braathen L. (2002): Photodynamic therapy using topical methyl 5-aminolevulinate compared with cryotherapy for actinic keratosis: A prospective, randomized study. *J.Am.Acad.Dermatol.* **47**: 258–262.

Szeimies R.M., Morton C.A., Sidoroff A., and Braathen L.R. (2005): Photodynamic therapy for non-melanoma skin cancer. *Acta Derm.Venereol.* **85**: 483–490.

Thompson M.S., Gustafsson L., Palsson S., Bendsoe N., Stenberg M., Klinteberg C.A., Andersson-Engels S., and Svanberg K. (2001): Photodynamic therapy and diagnostic measurements of basal cell carcinomas using esterified and non-esterified delta-aminolevulinic acid. *J.of Porphyrins and Phthalocyanines* **5**: 147–153.

Triesscheijn M., Baas P., Schellens J.H., and Stewart F.A. (2006a): Photodynamic therapy in oncology. *Oncologist.* **11**: 1034–1044.

Triesscheijn M., Ruevekamp M., Antonini N., Neering H., Stewart F.A., and Baas P. (2006b): Optimizing Meso-tetra-hydroxyphenyl-chlorin Mediated Photodynamic Therapy for Basal Cell Carcinoma. *Photochem.Photobiol.* **82**: 1686–1690.

Vakrat-Haglili Y., Weiner L., Brumfeld V., Brandis A., Salomon Y., McLlroy B., Wilson B.C., Pawlak A., Rozanowska M., Sarna T., and Scherz A. (2005): The microenvironment effect on the generation of reactive oxygen species by Pd-bacteriopheophorbide. *J.Am.Chem.Soc.* **127**: 6487–6497.

van den Akker J.T., Holroyd J.A., Vernon D.I., Sterenborg H.J., and Brown S.B. (2003): Comparative in vitro percutaneous penetration of 5-aminolevulinic acid and two of its esters through excised hairless mouse skin. *Lasers Surg.Med.* **33**: 173–181.

Vinciullo C., Elliott T., Francis D., Gebauer K., Spelman L., Nguyen R., Weightman W., Sheridan A., Reid C., Czarnecki D., and Murrell D. (2005): Photodynamic therapy with topical methyl aminolaevulinate for 'difficult-to-treat' basal cell carcinoma. *Br.J.Dermatol.* **152**: 765–772.

Wadhera A., Fazio M., Bricca G., and Stanton O. (2006): Metastatic basal cell carcinoma: a case report and literature review. How accurate is our incidence data? *Dermatol.Online.J.* **12**: 7.

Wallner K.E., Galicich J.H., Krol G., Arbit E., and Malkin M.G. (1989): Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma. *Int.J.Radiat.Oncol.Biol.Phys.* **16**: 1405–1409.

Wang L., Jacques S.L., and Zheng L. (1995): MCML—Monte Carlo modeling of light transport in multi-layered tissues. *Comput.Methods Programs Biomed.* **47**: 131–146.

Warloe T. (1995): *Photodynamic therapy of human malignant tumors, A study of epithelial tumors of the skin, gastrointestinal tract and malignant pleural mesothelioma*. The University of Oslo. (Ph.D. thesis).

Weersink R.A., Forbes J., Bisland S., Trachtenberg J., Elhilali M., Brun P.H., and Wilson B.C. (2005): Assessment of cutaneous photosensitivity of TOOKAD (WST09) in preclinical animal models and in patients. *Photochem.Photobiol.* **81**: 106–113.

Weishaupt K.R., Gomer C.J., and Dougherty T.J. (1976): Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a murine tumor. *Cancer Res.* **36**: 2326–2329.

Wennberg A.M., Larkö O., Lönnroth P., Larson G., and Krogstad A.L. (2000): Delta-aminolevulinic acid in superficial basal cell carcinomas and normal skin—a microdialysis and perfusion study. *Clin.Exp.Dermatol.* **25**: 317–322.

Wiegell S.R., Stender I.M., Na R., and Wulf H.C. (2003): Pain associated with photodynamic therapy using 5-aminolevulinic acid or 5-aminolevulinic acid methylester on tape-stripped normal skin. *Arch.Dermatol.* **139**: 1173–1177.

Wiegell S.R. and Wulf H.C. (2006a): Photodynamic therapy of acne vulgaris using 5-aminolevulinic acid versus methyl aminolevulinate. *J.Am.Acad.Dermatol.* **54**: 647–651.

Wiegell S.R. and Wulf H.C. (2006b): Photodynamic therapy of acne vulgaris using methyl aminolaevulinate: a blinded, randomized, controlled trial. *Br.J.Dermatol.* **154**: 969–976.

Wilson B.C., Patterson M.S., and Lilge L. (1997): Implicit and explicit dosimetry in photodynamic therapy: a new paradigm. *Lasers Med.Sci.* **12**: 182–199.

Wolf P. and Kerl H. (1995): Photodynamic therapy with 5-aminolevulinic acid: a promising concept for the treatment of cutaneous tumors [editorial; comment]. *Dermatology* **190**: 183–185.

Zhu T.C., Dimofte A., Finlay J.C., Stripp D., Busch T., Miles J., Whittington R., Malkowicz S.B., Tochner Z., Glatstein E., and Hahn S.M. (2005): Optical properties of human prostate at 732 nm measured in mediated photodynamic therapy. *Photochem.Photobiol.* **81**: 96–105.